

Synergistic Signals in the Environment

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Numerous endogenous signals (such as hormones and growth factors) or environmental signals (including chemicals or temperature) contribute to determining the overall biological response produced by cells. Some combinations of endogenous or environmental signals produce synergistic activity. This commentary examines the different types of interactions between signals that contribute to synergy at the biological level. *Key words:* environmental estrogens, hormones, synergy, temperature, turtles. *Environ Health Perspect* 104:1020–1023 (1996)

Some environmental chemicals exhibit hormonal activity when tested with *in vitro* assays and whole animal models. This activity appears to occur through the interaction of chemicals with a steroid hormone receptor, such as the estrogen receptor. The presence of hormonally active chemicals has drawn attention to their potential impact on wildlife and humans (1). The reduced phallus size of alligators in Lake Apopka, FL, (2) and the feminization of male fish in the United Kingdom (3) have been two of the most cited examples of the effects of hormonally active environmental chemicals on wildlife. Because of these observations, research has been focused on the relationship between environmental chemicals with hormonal activity and human disease. The concentration of environmental chemicals with weak estrogenic activity measured in women with breast cancer has been associated with this disease in some studies but not in others (4). The much-debated decrease in semen quality in men has been speculated to be linked to environmental estrogens (5). While some reports of adverse health effects in wildlife appear to be due to exposure to environmental chemicals with hormonal activity, the association of these chemicals with human disease remains inconclusive.

To study environmental chemicals that mimic natural hormones, we must understand the biology and toxicology of these environmental hormones and develop assays to identify chemicals with hormonal as well as antihormonal activity. Numerous laboratories are currently identifying these environmental chemicals and determining the concentrations required to produce hormonal responses using *in vitro* assays and whole animal models (1). This line of investigation has focused mostly on identifying chemicals with estrogenic activity, for example, bisphenol A, a chemical released during autoclaving polycarbonate products (6); hydroxylated polychlorinated biphenyls (PCBs), which are present in adhesives, fire retardants, and waxes (7); and phthalate

plasticizers, which are used in the production of plastics (8). The affinity of most environmental estrogens for the estrogen receptor (ER) is low, usually between one-fiftieth to one-ten thousandth that of the natural hormone estradiol (6–8).

While the identification of hormonally active chemicals is an important priority, the mechanisms by which these chemicals work has yet to be adequately elucidated. Our laboratory has shown that some synthetic or environmental estrogens have a lower affinity for extracellular binding proteins (e.g., sex hormone-binding globulin) in serum than estradiol (9). For example, the estrogenic activity of diethylstilbestrol (DES) is greater than an equal concentration of estradiol in the presence of sex hormone-binding globulin. The affinity of both chemicals for the human ER (hER) is similar, indicating that DES has a lower affinity for certain binding proteins than estradiol. This observation suggests that the intracellular concentration of some environmental estrogens may be increased relative to estradiol and effectively promote their functional interaction with the ER. Indeed, the weak environmental estrogens *o,p'*-DDT or octophenol are weakly bound to serum proteins and are, thus, more DES-like in their cellular distribution (9).

Another issue that has yet to be investigated is the potency of combinations of environmental chemicals in hormonally responsive cells. We recently explored the functional interaction of chemical mixtures based on measured levels of chemicals identified in alligator eggs from Lake Apopka, FL, which contained dieldrin (630 nM), chlordane (220 nM), toxaphene (200 nM), Aroclor 1242 (530 nM), *p,p'*-DDE (18 µM), *p,p'*-DDD (2.6 µM), *trans*-nonachlor (250 nM), and *cis*-nonachlor (160 nM) (10). The effect of mixtures of chemicals was addressed using molecular biology techniques to design *in vitro* assays to measure the estrogenic activity of selected chemicals identified in alligator eggs alone or in combination. Yeast

and human endometrial cancer cells were genetically engineered to produce the hER and to contain an estrogen-sensitive reporter gene.

Exposure of the yeast to the pesticides endosulfan, dieldrin, or toxaphene individually produced only a partial estrogenic response, even at micromolar concentrations (11). This is consistent with the observations that these environmental chemicals have an affinity for the ER that is one-ten thousandth that of estradiol. On the other hand, a combination of two chemicals, such as endosulfan and dieldrin, produced estrogenic activity that was between 150- and 1500-fold greater than the activity of either chemical alone. In fact, chlordane alone had no estrogenic activity in yeast, but when combined with endosulfan, dieldrin, or toxaphene, it significantly increased the estrogenic activity. These results demonstrated that two chemicals can produce greater than additive or synergistic estrogenic activity.

Since we and others have shown that different signaling pathways can be involved in estrogen action (12,13), it was important to determine if the chemicals interacted with the hER. An *in vitro* competition binding assay demonstrated a mixture of two chemicals, including chlordane, displaced radiolabeled estradiol in a synergistic manner (11). The ability of the hER to recognize two estrogens may occur through the presence of more than one binding site on the hER. This mechanism could potentially explain the structural diversity of chemicals with estrogenic activity.

While it appeared that a combination of two weak environmental estrogens produced estrogenic activity in yeast in a synergistic manner, even at the highest concentrations tested, the estrogenic activity of a combination of two chemicals was only 60% that of estradiol. It is unclear if the level of estrogenic activity induced by a combination of chemicals in yeast would be high enough to produce physiological estrogenic responses in mammalian cells and whole animal models. Furthermore, we have not tested a mixture of all the chemicals identified in the alligator eggs; thus, the net hormonal activity of the chemicals in the egg is not known.

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The pesticides used in our study are no longer in use in the United States, with the exception of endosulfan; however, some pesticides remain in the environment many years after their use has been discontinued. This can occur through the processes of bioaccumulation and biomagnification in plants and animals. Nonetheless, we believe it will be important to test modern-use chemicals that have hormonal activity to determine whether they also synergize. We are also investigating the possibility that combinations of chemicals can function as antihormones in a synergistic manner.

The results for endosulfan, dieldrin, toxaphene, and chlordane were obtained using yeast genetically engineered to express the hER. Yeast do not normally express steroid hormone receptors, but molecular biology techniques have allowed the introduction of steroid hormone receptors into yeast (14,15). Yeast that contain steroid hormone receptors have been used for mechanistic studies of these receptors because the assays can be performed inexpensively and rapidly and the conditions surrounding the receptors can be tightly controlled. Reports have shown that the hER expressed in yeast has affinity for estradiol that is similar to the hER expressed in mammalian cells. However, there have been reports that the antiestrogens tamoxifen and ICI 164,38, have estrogenic activity in yeast at high concentrations (15). Furthermore, Metzger et al. (16) have reported that the regions of the hER that are important for activating the transcription of estrogen-sensitive reporters in yeast and mammalian cells may be different. These regions of the hER are not believed to be involved in binding estrogens. Another area in which knowledge is limited is the metabolism of environmental chemicals with estrogenic activity in yeast. To address the potential differences with the ER in yeast and mammalian cells, our laboratory is currently conducting experiments with endosulfan and related compounds to determine if the results obtained in yeast can be reproduced using mammalian cells and whole animal models. While the results with endosulfan and related compounds in yeast are striking evidence of a synergistic effect of environmental hormones, it remains to be determined how accurately the results obtained in yeast predict the activities of these chemicals in mammalian cells and animal models.

This issue was partially addressed in another aspect of our study dealing with the estrogenic activity of hydroxylated polychlorinated biphenyls, 2',4',6'-trichloro-4-biphenylol (4-OH PCB) and 2',3',4',5'-tetrachloro-4-biphenylol (3-

OH PCB) (11). A mixture of the PCBs in the yeast system produced a sevenfold greater estrogenic activity than the individual PCBs. Again, this effect was shown in the competition binding assay to be apparently mediated by the interaction of the hER with both chemicals. In contrast to the chemicals described earlier, mixtures of PCBs were also assayed for estrogenic activity in human endometrial cancer cells expressing the hER. The synergistic response observed in yeast with mixtures of PCBs was also observed in the endometrial cells. While the observation of synergy in mammalian cells adds credibility to the observations in the yeast, it is possible that the estrogen-sensitive reporter used in the yeast and mammalian cells contributed to the synergy. The reporter used in both systems contained two consecutive binding sites (estrogen response elements) for the ER, which has been shown to produce synergistic activity compared to a reporter with one ER binding site in the presence of estradiol (17). We are now testing the possibility that the degree of synergy observed by combinations of chemicals is related to the number of ER binding sites on the reporter.

The human endometrial cancer cells used in this study were devoid of hER but were derived from a parental cell line that contained hER. The loss of hER expression in these cells may also indicate that certain proteins which are critical for hER functioning are also not expressed. Furthermore, the transient expression of the hER may function in these cells differently than in cells that contain endogenous hER. While the experiments in mammalian cells may be construed as better predictors of *in vivo* effects than the experiments in yeast, the work on the synergistic activity of the PCBs is supported by a series of whole animal observations done in collaboration with the laboratory of David Crews at the University of Texas.

The Crews laboratory has been studying the influence of temperature and estrogen on the sexual development of turtle embryos (18). The gender of red-eared slider turtles is determined by the temperature at which the eggs are incubated. At 26°C or 31°C, the embryos develop into males or females, respectively. The sex of male-determined turtle embryos can be reversed by painting the eggs with estradiol. Thus, estradiol can mimic the effect of temperature on the sex determination of turtle embryos. From this observation, it was hypothesized that the turtle embryos could be used to determine whether chemicals functioned as estrogens *in vivo* and to determine their effective activity relative to

estradiol. Treatment of male-determined turtle eggs with the estrogenic 3-OH and 4-OH PCBs produced sex reversal, but at concentrations higher than estradiol (19). Interestingly, a combination of the PCBs was effective in sex reversal at concentrations of the chemicals that had no activity alone. The experiments with PCBs in turtle eggs suggested that synergistic estrogenic activity was possible *in vivo* and identified an assay for measuring these effects.

While the synergistic activity of environmental chemicals may be occurring through one pathway, synergy can also be thought of as an integration between two or more pathways. The definition of synergy is twofold: a response that is greater than additive and an integration between different parts. Thus, synergy has both qualitative and quantitative aspects that may be coordinated in one congruent process. The activity of environmental chemicals functioning through the hER is an example of a response that is greater than additive. The integration between what had previously been considered to be distinct pathways has been elegantly illustrated by studies on the effects of temperature and hormones on the gender of turtle embryos.

The Crews laboratory has identified synergy between an environmental factor, heat, and an endogenous factor, estrogen (18). The temperature of 28.2°C produces mostly male turtles, but it is also the transition temperature between male and female differentiation. Eggs incubated at 28.2°C require substantially less estradiol for sex reversal than eggs incubated at 26°C, indicating that the increase in temperature and the amount of estradiol interact in a synergistic manner.

Other examples of the interaction of heat and the estrogen response may be illustrative of a role for synergy in hormone action. Picard et al. (20) demonstrated that the expression of the ER in yeast defective in a homolog of heat shock protein 90 resulted in reduced estrogen response compared to the wild-type strain. Heat shock protein 90 has been shown to be associated with hER prior to estrogen binding and, as such, may regulate the conformation of unoccupied hER. While the actual mechanism for the effect of temperature-dependent pathways on hormone action awaits further investigation, the interactions between heat and hormones are examples of the integration of two distinct pathways to produce a synergistic response.

Another example of the integration of diverse pathways to produce synergy involves the heat shock response in mammals. The activation of the heat shock

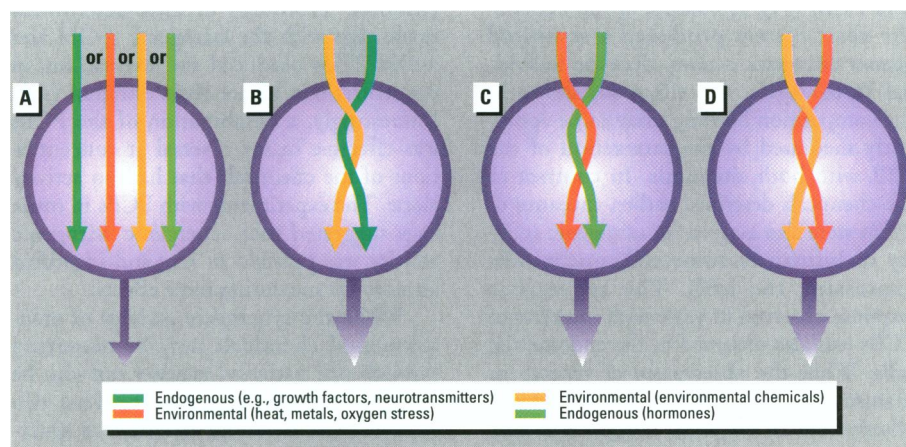


Figure 1. Modulation of cellular responses by synergistic combinations of environmental and endogenous signals. (A) The interaction of a cell with an endogenous or an environmental signal results in the production of a response; (B,C) the interaction of a cell with a combination of environmental and endogenous signals; (D) a combination of two environmental signals. B,C, or D may result in a synergistic response.

response pathway normally occurs at 42°C and involves the activation of heat shock transcription factors that regulate the expression of stress-response genes. Pretreatment of cells with arachidonic acid, a precursor to prostaglandin, reduced the temperature required for induction of the response to 39–40°C (21). This effect was also observed with the nonsteroidal anti-inflammatory drug indomethacin (22). Conversely, heating the cells to 40°C reduced the concentration of indomethacin required to induce heat shock gene expression in a greater than additive fashion.

Synergy has also been observed in hormone-response pathways involving endogenous signals. Various steroid hormone receptors have been shown to be transcriptionally activated following treatment of cells with the neurotransmitter dopamine or with growth factors such as epidermal growth factor (EGF); the transcriptional activation of the steroid hormone receptors is similar to that produced by the cognate hormone (12,13). Submaximal concentrations of a combination of dopamine or EGF and estradiol produced a synergistic increase in ER-mediated activity. These interactions represent synergistic responses of both a multiplicative and integrative nature.

This commentary suggests the roles for synergy in mediating cellular responses to environmental and endogenous signals. Environmental signals can include chemicals, temperature, and oxygen stress. Hormones, growth factors, and neurotransmitters are some examples of endogenous signals. Synergy can result from a combination of two or more environmental or endogenous signals (Fig. 1). The synergistic estrogen response produced by a combination of two environmental chemicals

through the hER is an example of an amplification produced by environmental signals interacting with a single response pathway. Synergy can also result from an integration between distinct signaling pathways. This type of synergy is demonstrated by growth factors or neurotransmitters and hormones in the action of steroid hormone receptors or the interaction of indomethacin and temperature. A combination of environmental and endogenous signals can produce synergistic activity as shown by the effects of temperature and estrogens on the sexual development of turtle embryos.

On reflection, it seems that biological synergy in its two aspects may be more common than previously thought. Furthermore, it is clear that environmental signals can produce synergistic biological responses; it is also becoming apparent that the study of environmental signaling has opened new ideas on the functioning of endogenous signals. Thus, the field of environmental signaling will contribute to a greater understanding of both toxicology and biology.

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